Chapter 3

On-line capillary liquid-liquid electroextraction of peptides as fast pre-concentration prior to LC-MS

Based on
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Abstract

In this research paper we show that capillary electroextraction (cEE) is capable of fast on-line peptide concentration and that it can be coupled on-line to LC-MS to result in a fast and sensitive method. EE takes place when an electric field is applied in a two-phase liquid-liquid system. Sample ions in the organic phase migrate very fast into the aqueous phase and are concentrated in a small zone. In this work, cEE of peptides is developed and coupled on-line to LC-MS via a switching valve. Comparison of 10 min of cEE-LC-MS with a conventional LC-MS injection showed more than 100-fold increased peak heights. Good calibration curves of five model peptides in the range of 0.05 - 5 μM were obtained. The linearity was good (R^2 values between 0.984 and 0.996) and relative standard deviations ranged from 5 % at the highest to 25 % at the lowest concentration (n=3). The LOD of bradykinin, angiotensin I converting enzyme inhibitor and angiotensin I were in the low nM range. Analysis of a tryptic digest of eight model proteins resulted in more than 170 peptides, without bias for pI or hydrophilicity. Urine analysis is demonstrated, resulting in a LOD around 0.04 μM urine for tryptic cytochrome C peptides spiked to urine and an increase of 42% in the number of chromatographic peaks compared to conventional LC-MS. In summary, cEE-LC-MS is a fast electrophoresis-driven sample preconcentration technique that is quantitative, able to extract a wide peptide range and applicable to bioanalysis.

1. Introduction

For several decades it is known that applying an electric field in solvent extraction procedures enhances mass transfer from one phase into the other. This process is sometimes referred to as two-phase electrophoresis [1-5], but more conveniently called 'electroextraction' (EE), a term that was first proposed in the early 1990s by Stichlmair et al [6]. Initially, EE has been developed as a purification technique in the field of chemical engineering, to enhance product yields [1-5, 7, 8]. However, around a decade ago Van der Vlis *et al.* adapted liquid-liquid EE for analytical purposes, using ethyl acetate (EtOAc) as organic phase and performing EE of test solutions in capillaries (cEE) [9-11]. Despite the promising results that were presented in these publications, analytical cEE has not been developed further since and no bio-analytical applications have been published. Recently, the potential of EE and related techniques received renewed attention via review papers on electrochemically modulated extraction methods [12] and electric field-amplified transport across phase boundaries [13].

When an electric field is applied in a two-phase liquid-liquid system that consists of a low conductive organic phase and a high conductive aqueous phase, charged compounds that are in the organic phase will migrate fast towards the aqueous phase. As soon as the aqueous phase is entered, migration speed decreases dramatically, causing analyte concentration. EE offers enhanced selectivity, as either cations or anions are extracted, while neutral compounds will largely remain behind since they only migrate to the aqueous phase by passive diffusion. In this set-up, only the electric field on the low-conductivity side of the interface will be of importance for ion migration [8]. To achieve a sample concentration effect, the analytes should therefore dissolve in the organic phase. A prerequisite of the organic phase is that it has some conductivity to enable ion transfer. Pure EtOAc hardly conducts current and therefore is not suitable for EE. However, when saturated, EtOAc contains 3.5% (v/v) water, which allows the presence of ions. The solubility of the small protein egg white lysozyme has been reported to be good (10 mg/mL) in EtOAc, [14] and some enzyme activities are even enhanced in an organic environment [15].

The major difference between cEE and conventional field amplified sample stacking (FASS) [16-19] is, thanks to the using of two immiscible liquid phases, the presence of a liquid-liquid interface. The liquid-liquid interface limits the influence of thermal currents caused by convection. When only one phase is present, as in FASS, thermal currents caused by convection can occur that frustrate the concentrating effect, while in a two-phase

system convection is constrained to a single phase by a physical border [6]. Therefore, zone broadening during EE is limited [6, 8]. Moreover, since very high electric fields strengths occur in the organic phase, EE is fast.

In this research paper, the cEE process was characterised by studying the current behaviour using ammonium as model cation and crystal violet for visualisation. Then, cEE of peptides was developed and coupled to LC-MS. The resulting cEE-LC-MS procedure was successfully applied to the analysis of a tryptic digest of several model proteins and urine. To our best knowledge, the application of cEE to biomolecules in complex biological samples has not reported before in literature.

2. Experimental

2.1 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water. Acetonitrile (ACN), EtOAc and formic acid (FA) were obtained from Biosolve (Valkenswaard, The Netherlands), ammonium acetate, ammonium bicarbonate, crystal violet, model proteins, proteomics grade trypsin, dithiothreitol, iodoacetic acid and glycine from Sigma (St. Louis, MO, USA), acetic acid and sodium hydroxide from JT Baker (Philipsburg, NJ, USA) and the model peptides (bradykinin 1-5, mass 572; bradykinin 1-6, mass 659; angiotensin I converting enzyme inhibitor (alias [Des-Pro2]-Bradykinin), mass 963; thymopentin, mass 679; bradykinin, mass 1060; angiotensin I, mass 1296 and substance P, mass 1347) from Genscript Corporation (Piscataway, NJ, USA).

2.2 Equipment and techniques

2.2.1 First EE set-up

To study the EE process, experiments have been carried out in a custom-made EE device (Fig. 3.1A). This device consisted of a vial (height: 108 mm, ID: 15 mm, material: polychlorotrifluorethylene (PCTFE), thickness 3 mm) with a conical bottom (angle 28˚) with two threaded inlets where a platinum electrode and a capillary were fixed with finger-tight nuts (Standard Head Fitting for 360 µM OD tubing, Upchurch Scientific, Oak Harbor, WA, USA). The capillary (OD: 400 µm, ID: 320 µm, material: fused silica) served to inject the aqueous phase below the organic phase using a 500 µL micro syringe (Kloehn

Ltd., Las Vegas, NV, USA). The bottom electrode was grounded. The conical bottom enabled extraction into a small aqueous phase volume. The electric circuit was closed with a gold coated anode (to minimise reduction/oxidation reactions) with a flat circled end contacting exactly the whole surface of the organic phase, ensuring all ions to be exposed to the electric field. High voltage was applied with a Spellman High Voltage Power Supply (Spellman, Hauppauge, NY, USA). Electric current was measured with a simple multimeter (Voltcraft, Oldenzaal, The Netherlands) and conductivity with a conductivity meter from Hanna Instruments (type HI 8733, Woonsocket, RI, USA).

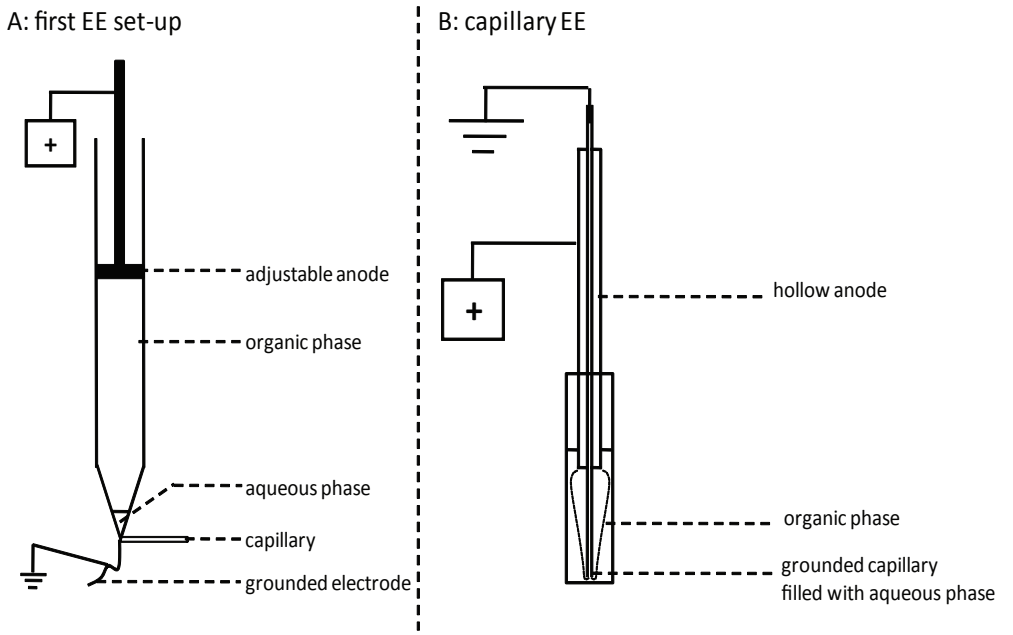

Figure 3.1 A) Schematic set-up of EE in the laboratory made device. B) Sample introduction set-up of capillary EE. The dotted line indicates the boundary of the effective extraction volume

Samples taken prior to and after EE were freeze-dried in a Zirbus Vaco I freeze-dryer (Zirbus Technology, Bad Grund, Germany) under vacuum that was applied with an Alcatel 2008A vacuum pump (Alcatel Vacuum Products, Hingham, MA, USA).

2.2.2 cEE-MS

cEE was carried out in an Agilent Technologies 1600 series CE apparatus (Agilent Technologies, Santa Clara, CA, USA). An untreated fused silica capillary (65 cm, 75 μm ID)

was filled with aqueous phase and dipped via a hollow electrode in the organic phase that contained the charged analytes. The organic phase (50 µL) was in a flat-bottom glass insert vial of 30.75 x 5 mm (Micro-insert, Bester BV, Amsterdam, The Netherlands). The capillary outlet was grounded via the spray source, while the organic phase was in contact with the anode. No height difference between capillary inlet and outlet was present. The capillary inlet was located close to the vial bottom (inner diameter 3 mm) and the hollow electrode tip close to the liquid surface (Fig. 3.1B), so the electric field was present throughout a maximal part of the organic phase. The extraction volume was estimated to be 14 µL (a cone with a length of 6 mm and a base diameter of 3 mm) by was visually observing an extraction of crystal violet, which showed depletion in the expected area. MS experiments were carried out on a Bruker Daltonics MicrOTOF MS (Bruker Daltonics, Bremen, Germany). Coupling of cEE to direct infusion (DI) was achieved using a sheath-liquid assisted grounded spray source supplied by Agilent Technologies (part number G160760001). The sheath liquid (50%/50% (v/v) ACN and 0.2% FA, 3 µL/min) served to close the electric circuit and for good spray. Further spray settings were: end-plate offset -529 V, capillary voltage -1762 V, nebuliser gas pressure 0.4 bar, dry gas flow 4.0 L/min and dry temperature 200 °C. The recorded mass range was 200-1200 m/z.

2.2.3 Liquid chromatography

LC was carried out with an Agilent Technologies 1200 series µHPLC apparatus, equipped with a ZORBAX SB-C18 (5 µm, 150 x 0.5 mm) capillary HPLC column, also purchased from Agilent Technologies. A 20 µL/min gradient elution was employed (solvent A: 0.1% FA in water, solvent B: 0.08% FA in ACN), starting at 0% B and ending after 30 min at 35%, unless otherwise stated. Next, the column was conditioned for 5 min with 95% solvent B and 5 min with 100% solvent A. On each day, a blank run was performed first.

2.2.4 Interface for coupling cEE and LC

Coupling of EE with LC was facilitated with a bio-compatible 6-port 2-way switching valve (Cheminert® HPLC injection valve model C2-1246, Valco Instruments, Houston, TX, USA). The rotor consisted of polyaryletherketone (PAEK). In this switching valve, the liquid has no contact with metal parts, preventing short circuiting and unsafe working conditions. The valve was switched manually and therefore a polytetrafluorethene (PTFE) handle was used.

2.2.5 On-line cEE-LC-MS

Coupling of cEE-LC to the time-of-flight (TOF) MS detector was realised with a conventional electrospray needle that was provided with TOF-MS (Bruker Daltonics) part number EN8389). The spray settings were as described in section 2.2, except nebuliser gas pressure (1.0 bar) and dry gas flow (8.0 L/min) to cope with the higher flow rate. In the case of cEE-LC-MS of digested model proteins, a linear triple quad - ion cyclotron resonance - Fourier transform - mass spectrometer (LTQ-ICR-FT-MS) (Thermo, San Jose, CA, USA) served as detector, to facilitate peptide identification. LC-MS was achieved with a conventional electrospray device delivered by the manufacturer. Spray settings for LTQ-ICR-FT-MS were: sheath gas flow 15 L/min, source high voltage 3 kV, capillary temperature 200 °C, capillary voltage 30 V and tube lens voltage 155 V. During analysis, the MS continuously performed scan cycles in which first a high-resolution (R = 100 000) full scan (200-1200 *m/z*) in profile mode was acquired by the FT-MS, after which MS/MS spectra were recorded in centroid mode by the LTQ for the 3 most intense ions (isolation width, 4 *m/z*; normalised collision energy, 35%). Dynamic exclusion was enabled (repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 180 s; relative exclusion mass width, 5 ppm) as was charge state screening (q = 1-4).

2.2.6 Data analysis/protein identification

Identification of model peptides was based on matching of the known masses with the obtained mass information. When MS/MS was performed, a database search was performed with the software application Bioworks (version 3.3), using the following settings for peptide identification: $\Delta CN > 0.100$, Xcorr versus charge state $1 \geq 1.80$, $2 \geq 2.50$, $3 \geq 3.50$, peptide probability < 0.5 and $\Delta ppm < 5.0$.

2.3 Sample pretreatment

First, a model peptide mixture was diluted twice with 1 mM acetic acid and acidified to pH 3 with FA to ensure cationic peptides. Then, EtOAc was saturated with the sample, by adding as much as possible without allowing a two-phase system to form. Then, it contained 2.2 % (v/v). The maximal percentage of 3.5% (v/v) was not reached, most likely because some water was already present.

Cytochrome C (horse and human), hemoglobin (human), ribonuclease A (pancreatic bovine), lysozym (chicken) and bovine serum albumine were digested using sequence grade trypsin. Per protein, 100 µg was digested with 1 µg trypsin in 200 µL 100mM ammonium bicarbonate (pH 8.5). Prior to digestion (12 hours at 37 °C), the proteins were reduced with

dithiothreitol (4.5 mM) and alkylated with iodoacetic acid (10 mM), as prescribed in [20]. After adding FA (2% v/v sample concentration), the digested proteins were purified by SPE using TopTip© pipette tips (Glygen Corp., Maryland, US) filled with a small amount of Poros Reversed Phase (RP)-2 SPE material. The material was wetted with ACN, equilibrated with 2% aqueous FA and the sample was applied. The trapped peptides were washed with 2% aqueous FA and elution was achieved with 50 µL 70/30 mixture of ACN and 2% aqueous FA. Next, 6.25 µL of the eluent was mixed with 110 µL anhydrous EtOAc. 70% ACN is better miscible with pure EtOAc (5.6%) than water (2.2%). No precipitation was observed.

Freshly voided urine samples were obtained from adult, healthy volunteers (age 18 to 29), pooled, acidified with FA (2%), divided in aliquots and stored at -80 ˚C until analysis.

3. Results and discussion

3.1 Description of the electroextraction process

At first, the EE process was characterised using the laboratory-built device (Fig. 3.1A). EE is a process of ion fluxes, and therefore the EE process can be followed by monitoring the current. The organic phase consisted of 11 mL EtOAc saturated with 10 mM ammonium acetate which was first adjusted to pH 3 with FA. After saturation, the EtOAc contained 0.2 mM ammonium acetate and 0.55 nM FA. The aqueous phase consisted of 100 µL 10 mM ammonium acetate (adjusted to pH 3 with FA). These phases have a large difference in conductivity (0.3 µS/cm and 1150 µS/cm were measured in the donor and acceptor phase, respectively). Electric field strengths of 40 kV/m (2.5 kV over 6.25 cm), 24 kV/m (1.5 kV) or 16 kV/m (1 kV) were applied for 10 min at similar pH, temperature and phase ratios.

Ammonium was used as model cation to study EE, for practical reasons (available in large amounts, enabling measurable currents), and crystal violet (0.5 µM) was added to the donor phase for visualisation of the EE experiment. Due to their very low concentrations FA and crystal violet do not contribute significantly to the measured current. Dissociation constants of neutral acids and cationic acids can shift in different solvents. However, for ammonium this shift is not dramatic in various studied organic solvents such as dimethyl sulphoxide and propylene carbonate [21], so it can be assumed that ammonia will be protonated and therefore present as ammonium in EtOAc under these experimental conditions. In Fig. 3.2A, the current profiles are shown.

Initially, the current decreased unpredictably, but after the first minute, the current

decreased exponentially (established by fitting with exponential trend lines) until a stable low current (I_{rest}) was reached. Increasing the field strength resulted in a faster current decrease. It was observed that the crystal violet immediately started to migrate into the aqueous phase, giving rise to a sharp zone and a growing depleted zone at the cathode side in the organic phase. Within several minutes, the whole organic phase was depleted into a sharp aqueous zone. It was observed that the speed with which the depleted zone was formed decreased over time, while simultaneously the current decreased exponentially (Fig. 3.2A). In the depleted zone, the removal of ions has caused a lower conductivity and therefore a higher electric field strength. Since the applied voltage is constant, the electric field strength that is over the organic region that still contains ions will be lowered. As the depleted region grows, the electric field strength in the non-depleted zone is decreasing. As a consequence of this changing electric field distribution in the EE system, EE takes place in a non-linear manner, namely, as shown in Fig. 3.2, exponentially. In Fig. 3.3, the change of electric field strength in the different zones is depicted schematically. At the start of EE (I), a uniform electric field strength is present in the organic phase. As EE proceeds (II, III, IV), a depleted region with a high electric field strength grows. Simultaneously, a slightly decreasing electric field strength is present in the aqueous zone, due the increasing amount of ions. Since the conductivity difference between the aqueous phase and the organic phase is so large, the small change in the aqueous phase has no significant influence on the situation in the organic phase.

We assumed that the exponential current decrease that was observed can be described as:

1) $$I(t) = I_0 . e^{-\beta t} + I_{rest}$$

In this equation $I(t)$ is the current as function of time t, I_0 the starting current and I_{rest} the current that is let through by saturated EtOAc, and β represents the exponential coefficient with which the current decreases during EE, therefore called the extraction rate. As can be observed in Fig. 3.2A, I_{rest} reached a constant value. To determine values of β with different electric field strengths, $I(t)$ was differentiated and the differentiated current $I'(t)$, i.e. dI/dt, of the current profiles between 1 and 10 min was plotted versus $I(t) - I_{rest}$. The resulting slopes are the β values, since the differentiated current can be expressed as

2) $$I'(t) = -\beta I(t)$$

Since EE is a process of depletion, β is negative. The larger the absolute value of β, the higher the extraction rate. As can be observed in Fig. 3.2B, rather good linear curves are

obtained (R^2 values 0.99, 0.94 and 0.77 for 40, 24 and 16 kV/m respectively). Increasing the applied electric field strength increases the extraction rate. However, when electric field strengths above 40 kV/m were applied in this EE set-up, visible instabilities (fluid motion and droplet formation) occurred at the liquid-liquid interface, while the current became instable and EE failed, which has also been reported in literature [7, 8].

A comparison between different ionic strengths in the aqueous phase showed that I_0 as well as the exponential factor β by which the current decreases were not influenced (data not shown). The conductivity difference between the aqueous phase and the organic phase is so large, that a 10–fold change in the ionic strength of the aqueous phase has no measurable effect. In consequence, EE is robust towards ionic strength variations in the acceptor phase, in contrast to field-amplified sample stacking (FASS). When cEE is coupled to LC, the ionic strength of the mobile phase (which also serves as aqueous acceptor phase in cEE) does not affect the performance of the EE process.

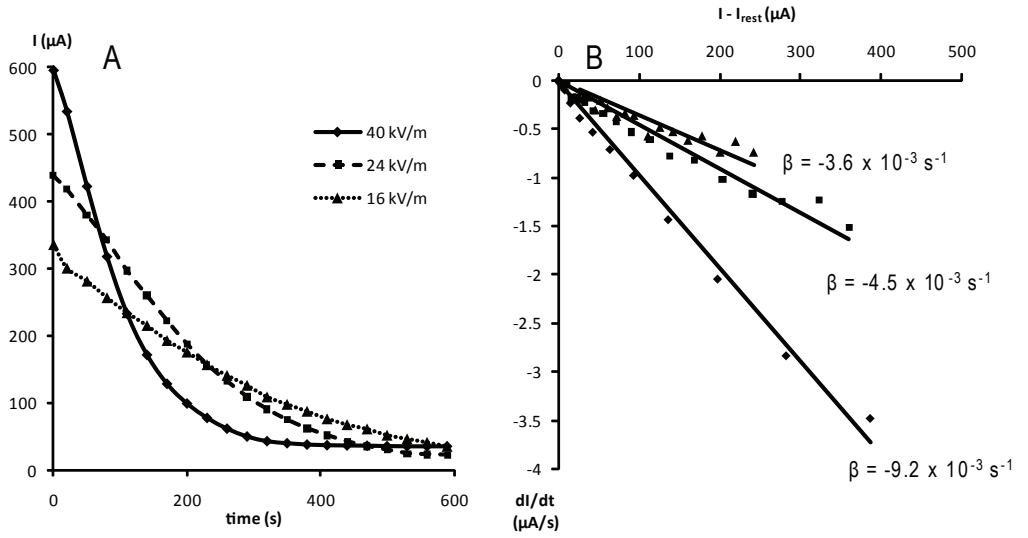

Figure 3.2 A) current profiles (n=3) of EE performed with 3 different electric field strengths B) dI/dt plotted against $I-I_{rest}$ of the current profiles shown in part A, to determine β values.

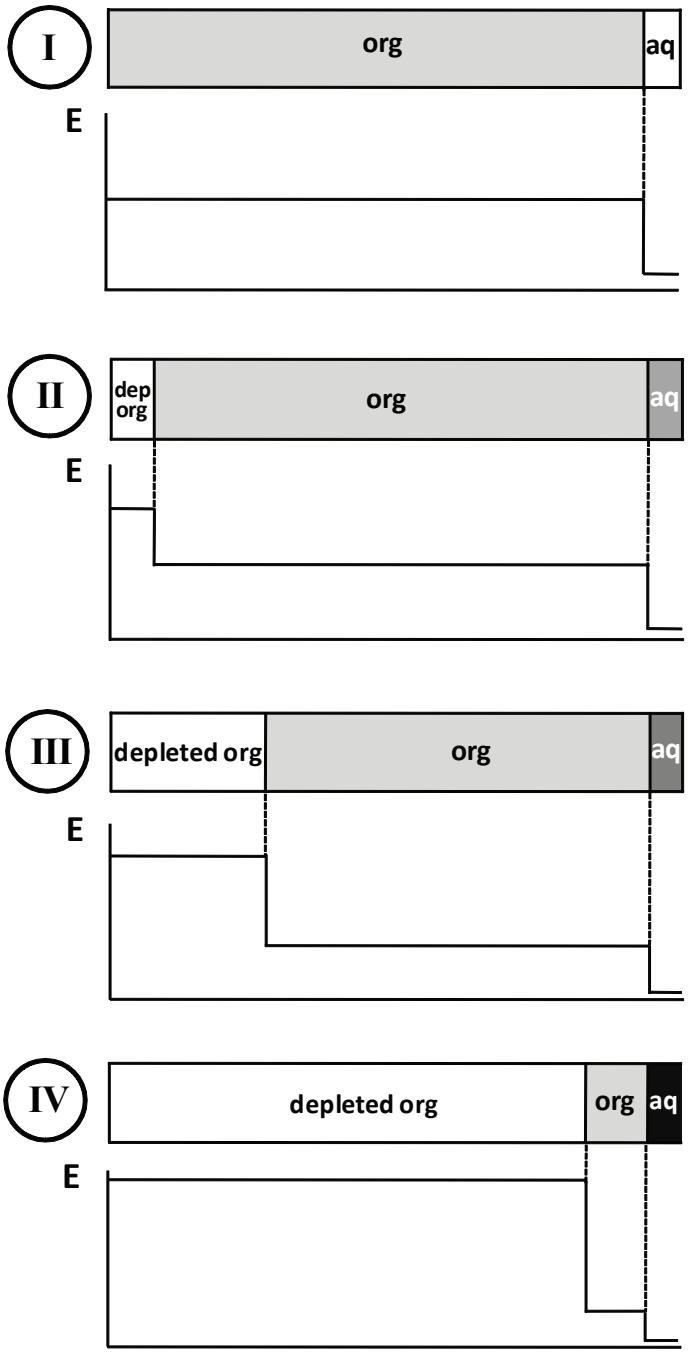

Figure 3.3 Schematic drawing of the changes in electric field strength during EE in the different zones. I, II, II and IV depict different time points in the process. During EE, the electric field strength in the organic zone decreases, while it increases in the depleted organic zone.

3.2 cEE of model peptides

A CE apparatus enables the handling of very small volumes and well-controlled high voltage and pressure application. Moreover, in a capillary, much higher electric field strengths can be used, since heat is dissipated more efficiently. This makes cEE an attractive option as selective sample injection method, as has been described before [9, 10]. On top of this, the influence of disturbances at the liquid-liquid interface can be expected to be smaller, since the area of the liquid-liquid interface is much smaller [7]. This partly compensates the fact that performing EE with a smaller area of the liquid-liquid interface is not advantageous for the speed of cEE. Moreover, much higher electric field strengths can be realised when using the CE apparatus. When 15 kV is applied, the electric field strength between the liquid-liquid interface and the electrode tip can be as high as 1.36×10^3 kV/m, which is more than 30 times higher than in the large-scale EE experiments.

A very high electric field strength exists in a short plug of low-conductive organic phase at the inlet of a capillary that is for the rest filled with higher conductive aqueous solution. The shorter the plug length and the larger conductivity difference between the zones will be, the larger the electric field strength will be, as was described by Chien and Burgi [17], who determined the optimal conditions for in-capillary sample concentration using field amplification.

Based on the above-mentioned aspects, a cEE procedure was developed. Theoretically, cEE speed is maximal when the liquid-liquid interface is exactly at the inlet of the capillary. However, due to interface instabilities (as described above) as well as EOF mismatch [18] it is difficult to fix the interface position at an exact location in the capillary. When the interface was too close to the inlet, cEE appeared to be unreliable. Therefore, a small aliquot of EtOAc was injected (approx. 2 cm, 3% of the capillary volume, i.e. ~88 nL). During cEE, this zone was slowly pushed back into the vial, using back-pressure, which also served to counter-effect the influence of the low EOF that still existed at pH 3. Optimisation of back-pressure, extraction time and extraction voltage resulted in the following injection scheme, analogous to Van der Vlis *et al* [9]: after a small EtOAc plug was injected by pressure (25 mbar) into the capillary, the first minute of cEE was carried out without back-pressure to let some more EtOAc enter the capillary by EOF. Then, a back-pressure of 25 mbar was applied during the remaining 9 min of the cEE procedure, to slowly push out the EtOAc plug without losing the concentrated sample zone. During EE, 15 kV was applied and the current profiles were monitored. Between measurements, the fused silica capillary wall was reconditioned by flushing 5 min with 0.1 M NaOH and 10 min with deionised water.

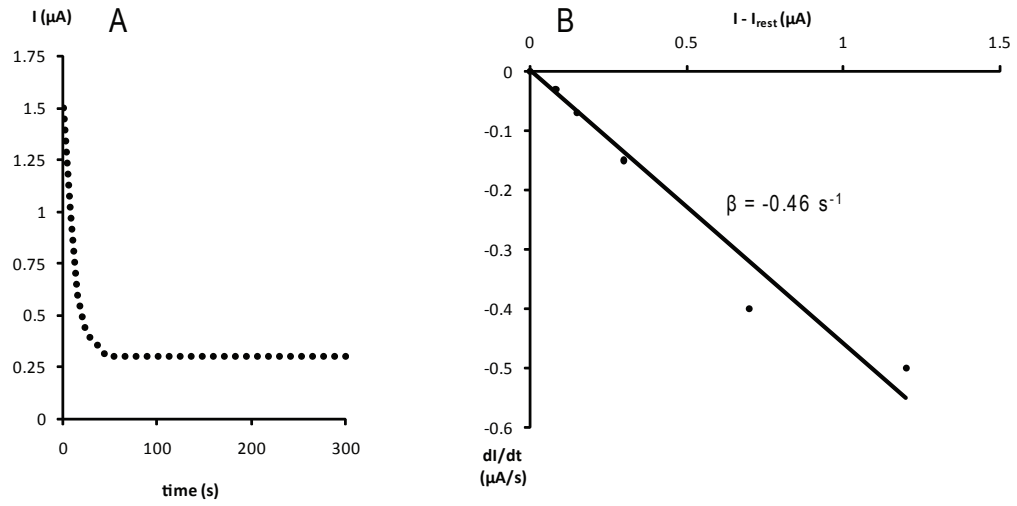

Figure 3. 4 A) cEE current profile of the extraction of 0.5 x 10^{-3} M ammonium (n=3) B) dI/dt plotted against $I-I_{rest}$ of the current profile shown in part A, to determine β values.

To study whether the cEE process is similar to the large-scale EE experiment discussed in the previous section, current behaviour during the extraction of 0.5 x 10^{-3} mol/L ammonium in EtOAc was monitored. Again, the current decreased exponentially (Fig. 3.4A), but much faster; the extraction rate β was much higher (Fig. 3.4B) compared to the first EE set-up. In Fig. 3.4A, it can be seen that during this period the current is stable and cEE is taking place without being disturbed. The current profiles were found to be reproducible.

Three consecutive cEE cycles of bradykinin, angiotensin I, bradykinin 1-5 and substance P (concentration in EtOAc 2.1 – 5 nM) were carried out without refilling or shaking of the sample vial. Since peptides have lower electrophoretic mobilities than ammonium, longer extraction times are needed to achieve peptide depletion, hence an extraction time of 10 min was chosen. After each cEE cycle, the sample zone was flushed to the MS using 50 mbar, for DI-MS analysis. After the first cEE, more than 96% of each peptide was extracted, while a consecutive extraction resulted in a 1 – 3% fraction, and a third consecutive extraction less than 0.1% (Table 3.1); percentages were calculated in relation to the sum of the peak areas of all three experiments. The total volume in the vial was 50 µL, but the electric field was not present throughout this whole volume (as discussed in section 2.2.2). This was confirmed by experiments in which a larger sample volume was used: peptide peak areas did not increase. On top of this, after mixing of the electroextracted sample, a consecutive cEE experiment

resulted again in high peptide peaks (data not shown). When the effective extraction volume is depleted, only peptides that slowly diffuse into the electric field zone are extracted. This is only a small amount of peptide in one experiment, because diffusion is a slow process. Since only a portion of the organic phase is analysed in the cEE set-up, it is complicated to obtain correct recovery data. Altering the shape or position of the electrodes to ensure that the electric field is present throughout the whole volume would be a solution, however, in the present experimental set-up using a commercial CE autosampler this is difficult to realise. Therefore, recovery was determined using the EE device described in section 2.2.1, where a sample of organic phase (100 µL) could easily be sampled before and after EE (10 mL EtOAc, 100 µL 10 mM ammonium acetate adjusted to pH 3 with FA as aqueous phase, 2.1 – 5 nM peptides, 10 min, 5 kV). The samples were evaporated, reconstituted in 100 µL 50%/50% (v/v) ACN/0.2% FA and analysed with DI-MS at a flow rate of 3 µL/min. Recoveries (based on measured ion intensities) were 77% for bradykinin, 90% for angiotensin 1, 80% for bradykinin 1-5 and 92% for substance P.

Table 3.1 Result of cEE of 4 model peptides. Percentages were calculated relative to the sum of the peak areas of the three consecutive experiments. In column of the 1st and 2nd cEE, the standard deviations are given (n = 3).

	peak area (%)		
peptide	**1st EE**	**2nd EE**	**3rd EE**
bradykinin	98.0 ± 1.4	2.0 ± 1.4	< 0.1
angiotensin I	98.8 ± 1.2	1.2 ± 1.2	< 0.1
bradykinin 1-5	97.7 ± 1.2	2.3 ± 1.2	< 0.1
substance P	96.6 ± 2.8	3.4 ± 2.8	< 0.1

3.3 Coupling of cEE to LC-MS

In [11], a cEE needle device was developed to couple cEE to LC. However, a switching valve offers a less complicated solution. A 6-port 2-way switching valve with an electrically isolated rotor and stator (see experimental section) was used (Fig. 3.5). This valve model has proven to be useful as interface for coupling LC-type systems to CE in several designs [22-24]. The cEE-LC-MS method includes (i) the cEE step; the valve is in the load position, the sample loop connects the inlet and outlet of the CE system; (ii) the sample transfer step; after cEE, the sample is flushed into the sample loop by applying pressure (~1000 mbar). The optimal flush time was determined to be 45 s. A rather large sample loop (5 µL) was chosen

to give the operator a suitable time window to manually switch the valve; (iii) the injection step; the valve is switched into the inject position and the sample is injected into the LC column. In future work, manual switching can be exchanged with automated switching. Band broadening due to the large sample loop volume and application of hydrodynamic pressure was overcome by starting the LC gradient with 1 min 100% 0.1% FA (solvent A), to allow for peptide trapping on the front of the column.

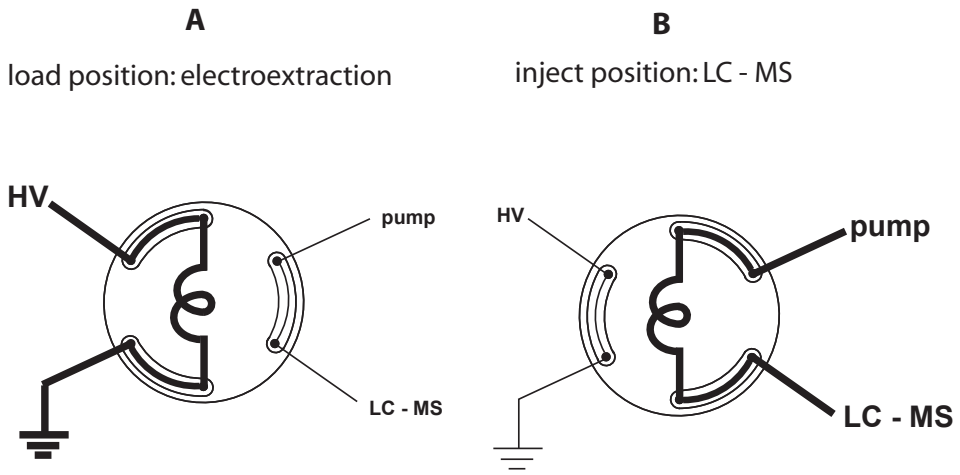

Figure 3.5 Schematic drawing of the coupling of cEE to LC. The bold line indicates the active part of the system. In part A, cEE of the analytes takes place, in part B, the concentrated analyte plug is injected into the LC-MS system

An LC-MS run and a cEE-LC-MS run of a test mixture of 5 model peptides (bradykinin 1-6, bradykinin 1-5, ACIE-bradykinin, bradykinin and angiotensin 1) were compared (Fig. 3.6). In the LC-MS run, 0.1 µL sample (5 µM per peptide in 70% ACN / 30% 0.1% FA) was injected, the recommended maximum for this column when the sample is not in solvent A (0.1% FA), as is the case after RP-SPE sample pretreatment. cEE-LC-MS resulted in 100 times increased peak areas using the same starting peptide concentrations, requiring only 10 min longer analysis time. Peak widths were not impaired and the relative peak heights basically remained the same. The LC injection volume was 0.1 µL, while cEE depletes maximal 14 µL (section 3.1). The peak heights were about 100 times increased, indicating approximately 70% depletion of 14 µL. However, in section 3.2, we showed that nearly complete depletion takes place, so apparently the volume where the electric field is present is somewhat smaller, i.e. about 10 µL, than expected.

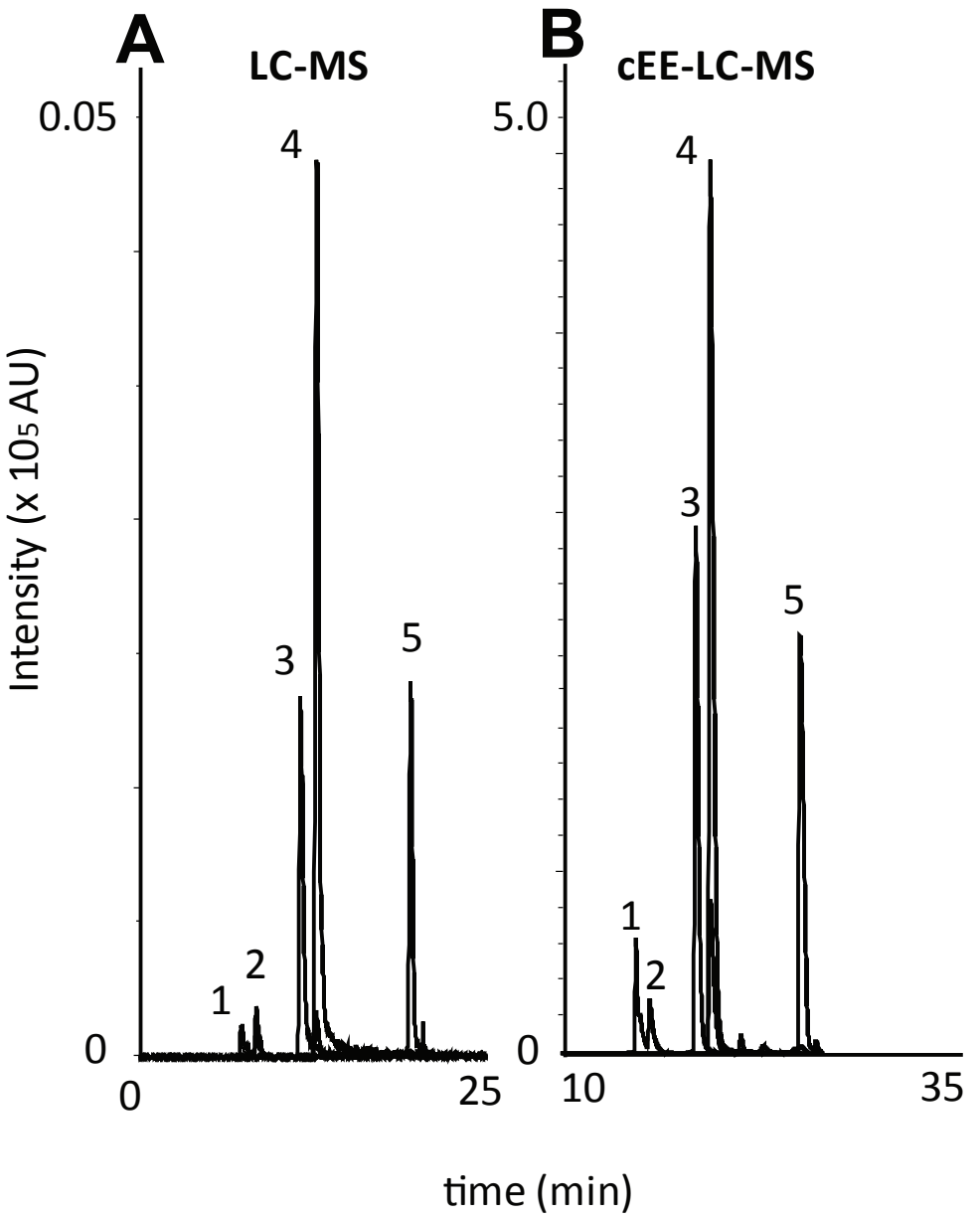

Figure 3.6 Typical example of the concentrating effect of EE. A) chromatogram resulting from conventional LC-MS and B) chromatogram resulting from cEE-LC-MS. When EE was performed, base peak heights were increased ~2 orders of magnitude compared to conventional LC-MS. 1) bradykinin 1-5, 2) bradykinin 1-6, 3) angiotensin I converting enzyme inhibitor , 4) bradykinin, 5) angiotensin I (AU = arbitrary units).

Calibration curves of electroextraction of 5 peptides from EtOAc were generated, containing 5 points (n = 3). As internal standard, thymopentin was used, while the concentrations of the 5 other peptides were varied between 0.05 μM and 5 μM in EtOAc, corresponding with injected sample amounts between 0.01 and 5 pmol. These levels are within biological range [25]. The linearity of cEE-LC-MS after internal standard correction is excellent (R^2 values between 0.984 – 0.996), the curve intercepts are significantly through zero (P values < 0.05) and the repeatability of the calibration curves is good (relative standard deviation of the curve slope between 0.73 and 12.0%) (Table 3.2). The relative standard deviations of the experimental values ranged between 4.6% at the highest concentration to 25.0% at the lowest concentration; the overall mean relative standard deviation of all the measurements was 14.7%. At the trace levels from 0.05 μM in EtOAc and below, reproducibility decreased dramatically. However, at 20 nM, bradykinin, angiotensin I converting enzyme inhibitor and angiotensin I were still detectable with a signal-to-noise ratio above 10. In consequence, for these peptides the LOD is estimated to be around 3 nM.

Table 3.2 Regression data of the calibration curves of 5 model peptide academic standards, P < 0.05 indicates intercept is through zero (n = 3).

Peptide	R^2	slope ± rel. st. dev (x 10^6 AU)	P-value intercept
bradykinin	0.996	15.5 ± 0.1%	0.00026
bradykinin 1-5	0.990	0.87 ± 7.9%	0.00026
bradykinin 1-6	0.984	1.03 ± 12.3%	0.000023
angiotensin I converting enzyme inhibitor	0.991	7.09 ± 1.9%	0.00031
angiotensin 1	0.988	27.5 ± 4.6%	0.0002

3.4 Applications

cEE can serve as a fast and simple interface between SPE and LC. Normally, RP-SPE eluents are freeze-dried and reconstituted in water, which is labour-intensive, time-consuming and may also introduce an additional error to quantitative results due to sample loss by evaporation, adsorption or reconstitution problems. cEE only requires mixing of the eluent with EtOAc and is capable of concentrating the analytes in a very small volume (< 1 μL) that can be handled accordingly. Moreover, as mentioned in section 2.3, the

eluent, containing 70% ACN, is better miscible with EtOAc than water (~5.5% instead of 2.2%), enabling a larger sample amount to be introduced for cEE. When attempting the first cEE experiments with biological samples, low recoveries were obtained. Probably, the peptides are not entirely in solution in cationic form in the EtOAc phase, possibly due to a precipitation reaction with matrix components. Possibly, adding extra components to the EtOAc will offer a remedy, this has our future attention.

3.4.1 Protein digests

A model protein digestion mixture containing human cytochrome C, horse cytochrome C, human haemoglobin, chicken lysozyme, bovine ribonuclease A and bovine serum albumin was analysed. Of each protein, the initial amount (prior to SPE), was 12.5 µg, corresponding with 0.18 - 1.0 nmol. The amount of digested protein present in one cEE experiment was maximal 0.023 - 0.13 nmol. Since the effective extraction volume was ~ 10 µL, the amount of protein that was actually extracted was maximal 2.1 - 12 pmol.

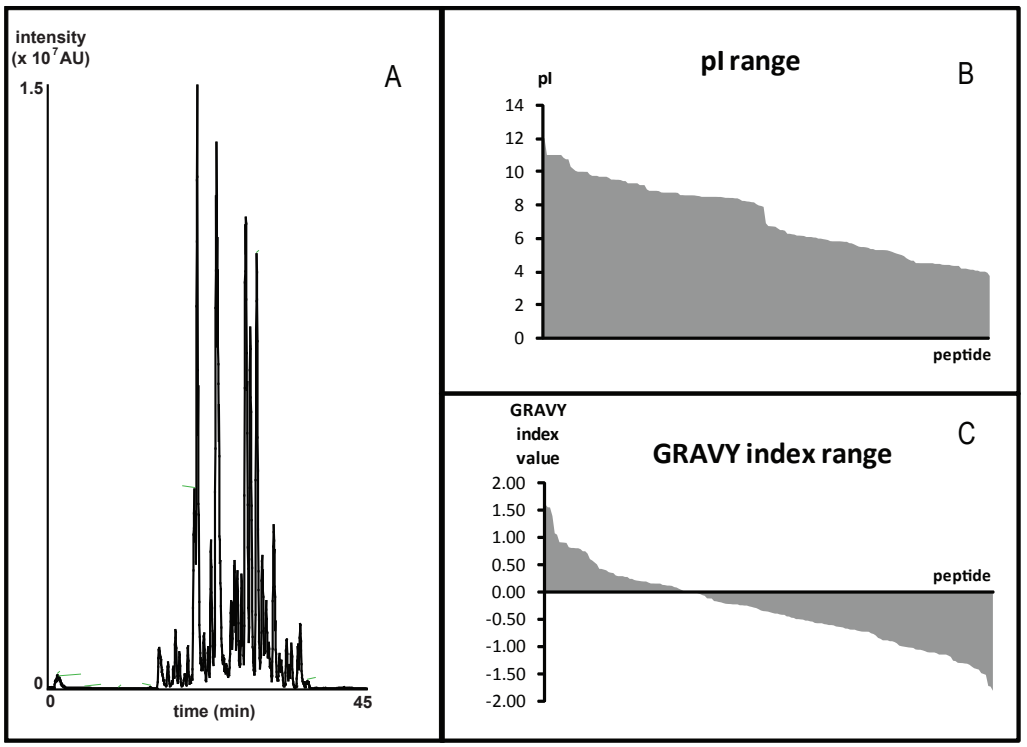

Figure 3.7 A) cEE-LC-ICR-FT-MS chromatogram of a model protein digest. Gradient: 0 to 50% B in 50 min. B) pI range of all extracted peptides and C) GRAVY index range of all extracted peptides

More than 170 peptides were extracted, separated and identified in one single run. The found peptide masses ranged from 500 to 4200 Da, 5 to 39 amino acids. Protein coverage was satisfactory: between 48% and 94 %, average 65%. As can be observed in Fig. 3.7, peptides with pI values ranging from 4 to 12 were extracted. The GRAVY index is a measure for the hydrophobicity of a protein, where lower values represent more hydrophilic peptides [26]. In Fig. 3.7, it can be observed that peptides above and below 0 were extracted. Studying the peptides that were not found back in this experiment (hence the protein coverage percentages below 100), revealed no different pI and GRAVY index range and pattern (data not shown).

3.4.2 Urine analysis

Fig. 3.8 shows a direct comparison between LC-MS and cEE-LC-MS of an SPE eluent (70% ACN) of urine spiked with 2 model peptides and cytochrome C digestion (0.5 µmol/L). In the LC-MS run, the eluent was diluted (5.5 %, i.e. 18 times) with water leading to the same analyte start concentrations as when cEE-LC-MS was performed. In a 1 µL LC-MS injection an aliquot of 0.2 µl urine was injected. In total, 96 peaks were detected, the highest being 1.35 x 10^4 AU (Fig. 3.8A). However, after cEE-LC-MS, a larger aliquot of approximately 3 µl urine could be injected and the peaks were around 15 times higher (the highest being much higher; 25 x 10^4 AU) and as much as 136 peaks were found (Fig. 3.8B), corresponding to an increase of 42% of the number of peaks. Probably, not all peaks represent peptides, but also a variety of endogenous metabolites.

To emphasise the potential of cEE-LC-MS in the field of peptidomics, a brief feasibility study of the extraction of peptides from urine was carried out. To aliquots of 200 µL, different amounts of horse cytochrome C digestion were added, resulting, assuming complete digestion, in peptide concentrations of 10 µM to 0.2 µM. Angiotensin I (2 µM) served as internal standard. The most abundant tryptic peptide, TGPNLHGLFGR, m/z 1169.62, could be detected even at the lowest concentration of 0.2 µM (2.5 µg/L) with a signal-to-noise ratio of ~50, corresponding to an LOD of this peptide around 0.015 µM. LOD values reported for LC-MS of peptides using similar columns and gradients, including evaporation of the SPE eluent, but larger injected sample aliquots, are between 0.065 and 1 µM [25]. The calibration curve (n = 3) showed excellent linearity (R^2 > 0.99), good reproducibility (relative standard deviation 14 %) and a recovery (of the whole method, thus including RP-SPE) of 56%. Moreover, several endogenous peptides were found, of which one could be identified to be bradykinin, which is a peptide known to occur in urine in a concentration between 10 and 35 nM [27].

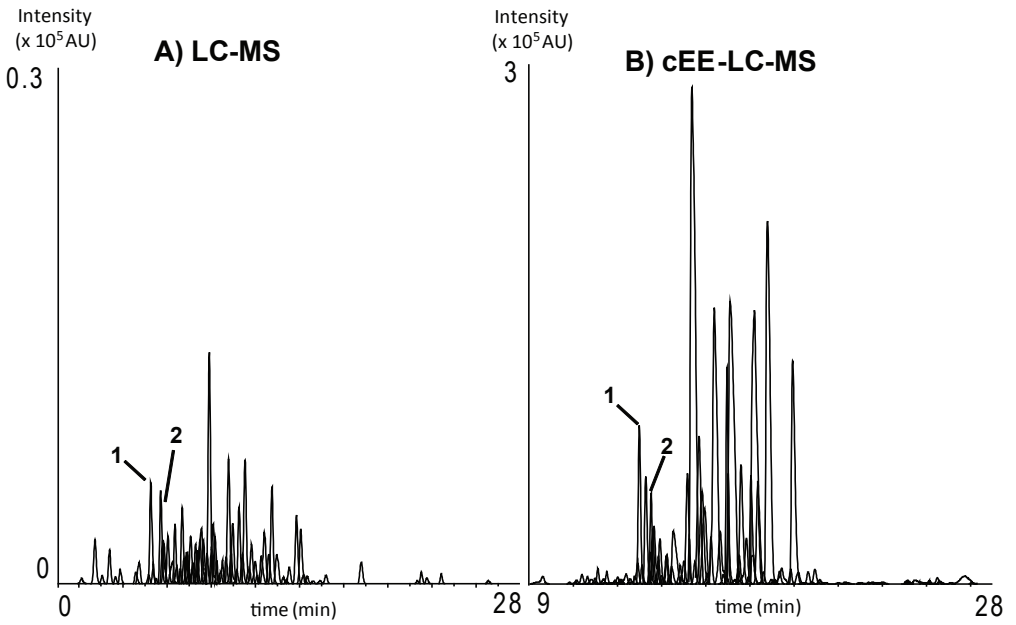

Figure 3.8 Extracted ion chromatograms of all peaks resulting from A) LC-MS with 1 µL injection, B) capillary cEE-LC-TOF-MS of urine spiked with cytochrome C digestion and internal standards after off-line SPE 1) cytochrome C peptide TGPNLHGLFGR, 2) angiotensin I. Other peaks are other cytochrome C fragments, putative endogenous peptides or other metabolites. Please note the difference of one order of magnitude between the y-axes. AU stands for arbitrary units.

In the cEE-LC-MS procedure used, an aliquot of approximately 3 µL urine is injected. Alternatively to cEE-LC-MS, the SPE-eluent (containing 70% ACN) can be injected directly. If the same urine aliquot as in the cEE-LC-MS experiments is then to be injected in a LC-MS system, 1 µL SPE eluent should be injected. An attempt to do this showed that this is not feasible (data not shown). This is because the elution strength of the SPE eluent (70% ACN) resulted in severely impaired separation on the used column (0.5 x 200 mm). It is possible to inject a higher volume of SPE eluent after it is sufficiently diluted with water, but this will not allow injecting an aliquot of 3 µL urine into this µLC system, where maximal 8 µL can be injected. One alternative would be evaporation of the eluent, but this results in the above mentioned disadvantages. Another alternative is to inject 3 µL untreated urine directly into the LC-MS system, but then high levels of contaminants are introduced that will interfere with separation and detection, leading to poor analysis and fouling of the systems.

4. Concluding remarks

The process of EE has been characterised and it has been demonstrated that the developed EE protocol is applicable to systems with different physical dimensions. The potential of cEE of peptides has been demonstrated using a set of model peptides. The peptides were concentrated two orders of magnitude from the organic into the aqueous phase. Moreover, the on-line coupling of cEE with LC-MS via a valve interface allows concentration, separation and detection of the concentrated analyte plug and minimises sample handling after, e.g., an SPE step. Linear response of the on-line cEE-LC-MS set-up has been obtained for model peptides. The analysis of a protein digest showed no bias to pI value or hydrophobicity. Furthermore, cEE of peptides was applied to a biological matrix. cEE can serve as a simple and fast interface between SPE and LC. Instead of evaporation and reconstitution of an SPE eluent, it can be mixed with EtOAc and injected into the LC system. In combination with an SPE step good LOD values, comparable to current published LC-MS methods (at low nM level), could be obtained for endogenous peptides in urine,. On-line coupling of SPE to LC via cEE has our future attention, as has an improved design of the cEE set-up to increase the effective extraction volume and improving reproducibility. In addition, we will optimise the SPE step. By enhancing the loadability of the method, i.e. enlarging the extraction volume, lower LOD values can be reached. We aim at the analysis of an entire SPE eluent in one experiment. For improvement of reproducibility, it seems a logical step to ensure that the effective extraction volume equals the total present organic phase, since turbulence of the organic phase and diffusion do not affect the extraction result anymore.

Next to peptides, metabolites have been detected in urine, suggesting that the EE approach is also suited for the analysis of metabolites.

5 . References

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